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FISSION YEAST MEDIATOR COMPLEX AND ITS ROLE IN TRANSCRIPTIONAL REGULATION

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ABSTRACT

Mediator is an evolutionary conserved co-activator complex that regulates transcription of protein encoding genes in eukaryotes. This multiprotein complex was first identified in budding yeast and shown to be indispensable for RNA polymerase II (pol II) dependent transcription. Mediator is a transducer of regulatory signals from gene specific transcription factors to the general transcription machinery. Mediator has both activating and repressive functions, but the regulatory mechanisms are not yet completely understood. In our work, we have used a biochemical approach and identified four proteins, Med12, Med13, Cdk8, and CycC, as subunits of a repressive kinase module present in the *Schizosaccharomyces pombe* Mediator complex. Taking advantage of a reconstituted *in vitro* transcription system, we have tried to address the mechanism of transcriptional repression by this subcomplex. We have also addressed the function of the Med15 Mediator subunit, which has been characterized previously in other species.

In Paper I we isolated a new form of the *S. pombe* Mediator complex to near homogeneity and denoted this complex Large (L-) Mediator. The L-Mediator contains four additional subunit not previously identified in the *S. pombe* core Mediator complex, but present in many of the large Mediator complexes isolated from higher eukaryotes. In contrast to the core Mediator complex, L-Mediator does not interact with pol II. Based on our biochemistry findings and bioinformatic analysis, our data suggest that Med12, Med13, Cdk8, and CycC form evolutionally conserved kinase module.

In Paper II we developed an *in vitro* transcription system reconstituted with all the general transcription factors purified to near homogeneity, in either native or recombinant form. Fission yeast pol II assisted by TFIIB, TFIIF, TFIIE, TFIIH, and budding yeast TBP could initiate transcription from the *S. pombe* adh1 promoter *in vitro*. We found that addition of the core Mediator in complex with pol II could stimulate basal transcription, while L-Mediator suppressed transcription initiation in a dose dependent manner. Based on our findings, we proposed a model explaining the repressive function of L-Mediator.

In Paper III we systematically addressed the structural organization of kinase module of L-Mediator. We identified Med13 as a key architectural subunit, anchoring the kinase module to the rest of the Mediator complex. Med13 was sufficient and necessary to occlude pol II from binding to Mediator, whereas deletion of Cdk8 and CycC did not affect Mediator association with pol II.

In Paper IV we identified two new *S. pombe* Mediator components. The evolutionary conserved Med15 subunit associated with the chromatin remodeling protein Hrp1 and formed transient interactions with the *S. pombe* L-Mediator complex. Genome wide association data demonstrated Med15 association with a distinct subset of Hrp1 bound gene promoters.

LIST OF PUBLICATIONS

I. Camilla O. Samuelsen, Vera Baraznenok, **Olga Khorosjutina**, Henrik Spåhr, Thomas Kieselbach, Steen Holmberg and Claes M. Gustafsson (2003) TRAP230/ARC240 and TRAP240/ARC250 Mediator subunits are functionally conserved through evolution.
Proc Natl Acad Sci USA. 98(21): 11985-90

II. Henrik Spåhr, **Olga Khorosjutina**, Vera Baraznenok, Tomas Linder, Camilla O. Samuelsen, Daniel Hermand, Tomi P. Mäkela, Steen Holmberg and Claes M. Gustafsson (2003)
Mediator influences *Schizosaccharomyces pombe* RNA polymerase II dependent transcription *in vitro*.
J Biol Chem 278(51): 51301-6

III. Vera Baraznenok*, **Olga Khorosjutina***, Zsolt Szilagyi, Camilla O. Samuelsen, Nina N. Rasmussen, Tomas Linder, Steen Holmberg and Claes M. Gustafsson (2011)
Molecular analysis of the Cdk8 module from Fission yeast L-Mediator.
Manuscript

IV. **Olga Khorosjutina**, Paulina H. Wanrooij, Julian Walfridsson, Zsolt Szilagyi, Xuefeng Zhu, Vera Baraznenok, Karl Ekwall and Claes M. Gustafsson (2010)
A chromatin-remodeling protein is a component of Fission yeast Mediator.
J Biol Chem 285(39) 29729-37

* These authors contributed equally to this work.

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LIST OF ABBREVIATIONS

ARC	activator recruited co-factor
bp	base pair
BRE	B-recognition element
CAK	cyclin activating kinase
Cdk	cyclin dependent kinase
CHD	chromodomain-helicase-DNA binding
ChIP	chromatin immunoprecipitation
C-Mediator	core Mediator
CRSP	co-factor recruited for SP1 activation
CTD	C-terminal domain
Cyc	cyclin
DPE	downstream promoter element
EM	electron microscopy
GTF	general transcription factor
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
Hrp	helicase related protein
IGR	intergenic region
Inr	initiator
kDa	kiloDalton
L-Mediator	large Mediator
MDa	megaDalton
Med	Mediator subunit
MTE	motif ten element
mRNA	messenger RNA
ORF	open reading frame
PIC	pre-initiation complex
pol II	RNA Polymerase II

Rpb	RNA polymerase B (II) subunit
S. cerevisiae	*Saccharomyces cerevisiae*
S-Mediator	small Mediator
S. pombe	*Schizosaccharomyces pombe*
SAGA	Spt-Ada-GCN5-acetyltransferase complex
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sp	*Schizosaccharomyces pombe*
Srb	suppression of RNA polymerase B(II) mutation
TAF	TBP associated factor
TAP	tandem affinity purification
TBP	TATA binding protein
p-TEFb	positive transcription elongation factor b
TFII	transcription factor assisting RNA polymerase II transcription
Trap	thyroid hormone receptor associated protein
UAS	upstream activating sequence

1 INTRODUCTION.

The array of expressed proteins determines the identity of the individual cell in a multicellular organism. Therefore the pattern and level of expression of a particular protein must be under tight control and as a consequence, expression of the corresponding gene has to be accurately regulated. Genes can be regulated at many different levels, e.g. gene transcription, splicing, mRNA stability, translation, protein stability, and post-translational modifications. However, in general, regulation at the level of transcription is quantitatively very important. The transcription process can be divided into three stages: initiation, elongation, and termination, which are subjected to regulatory mechanisms. Transcription initiation has been described as the most important of these levels, providing a heavily regulated, rate-limiting step of the gene expression cascade. Recently, our view of transcription initiation has changed, since there has been a series of papers demonstrating that RNA polymerase (pol II) may initiate transcription, but remain in a paused position downstream of the transcription initiation site, waiting for regulator input that signals a switch to elongation (Margaritis & Holstege, 2008).

A large number of different proteins and protein complexes with diverse enzymatic activities are needed for mRNA synthesis to occur in eukaryotic cells. These proteins include pol II and the general transcription factors that are required for transcription initiation at all genes; gene specific factors (activators and repressors) that help the transcription machinery to recognize the proper place for initiation of transcription (promoter) and initiate transcription; chromatin modifying and remodeling factors needed to make the chromatinized DNA template accessible for transcription factors binding and function; proteins that regulate the elongation; proteins required for processing of the primary transcript during elongation; and factors that stimulate transcription termination and help to transfer the transcript to the next step in the gene expression pathway.

In this thesis, we will primarily focus on the mechanisms of transcription initiation and discuss the role of the multiprotein Mediator complex in transcription regulation.

1.1 GENERAL TRANSCRIPTION MACHINERY.

Pol II is the enzyme responsible for transcription of all nuclear, protein-encoding genes in the eukaryotic cell. Pol II initiates transcription at the promoter element located

immediately upstream of the gene. The core promoter is defined as sequence directing accurate initiation of transcription by pol II (Juven-Gershon & Kadonaga, 2010). Studies on eukaryotic promoters have identified a number of promoter elements. One of these elements, the TATA box, is normally situated 25-35 base pairs upstream from the transcription start site in higher eukaryotes and fission yeast, but 40-120 bp in budding yeasts. A number of other eukaryotic core elements have also been identified and characterized, including BRE (B Recognition Elements), Inr (Initiator), DPE (Downstream Promoter Element), MTE (Motif Ten Element) (Juven-Gershon & Kadonaga, 2010; Thomas & Chiang, 2006). Another type of cis-regulatory DNA sequences situated upstream of promoters is involved in transcriptional regulation. These regions, often referred as Upstream Activating Sequences (UAS) or enhancers, are targeted by DNA binding proteins (gene specific transcription factors) that control the levels (activation or repression) and timing of transcription (Fuxreiter et al, 2008).

Pol II requires a set of additional general transcription factors to recognize a promoter and accurately start DNA dependent RNA synthesis. Pol II transcription starts with a controlled assembly on the core promoter of a set of transcription factors (TFIIB, D, E, F, and H) to form a so-called pre-initiation complex (PIC). These general transcription factors (GTFs) are not only needed for the promoter recognition, but also for promoter melting and the switch from the initiation stage to transcription elongation (Orphanides et al, 1996).

First, the multi-protein TFIID complex via its TBP subunit (for TATA binding protein) recognizes and binds the TATA box causing a sharp DNA bend and thus creating a context for binding of other transcription factors. Next, TFIIB is recruited via its C-terminal domain. TFIIB stabilizes the DNA/TFIID association, probably by assisting bending of DNA in the TBP-bound state and also recruits pol II via its N-terminal domain. Pol II might be either recruited alone or in association with TFIIF. Together, TFIID, TFIIB, and pol II form a minimal initiation complex that can transcribe a 3´-tailed template. TFIIF provides a scaffold for TFIIE and TFIIH binding, and recruitment of these two factors completes PIC formation. The enzymatic activities of TFIIH are required for DNA unwinding as well as phosphorylation of the C-terminal domain of pol II (CTD), an event that is essential for transcription initiation.

Stimulatory gene specific transcription factors (activators) bind to the promoter enhancer region, stimulate recruitment of the GTFs and pre-initiation complex assembly. In addition, there may be a need for factors that enable activator function, so

called co-activators, which may function as a bridge between activators and the general transcription machinery.

1.1.1 RNA polymerase II

Pol II is a large (more than 500 kD) multi-polypeptide complex composed of 12 subunits, denoted from Rpb1 to Rpb12, which are highly conserved throughout the eukaryotes. The degree of similarity between human and *S. cerevisiae* pol II is so high that half of human pol II subunits (hRpb5, hRpb6, hRpb7, hRpb8, hRpb9, hRpb10) can function in place of their yeast counterparts *in vivo*. The two largest pol II subunits (Rpb1, Rpb2) share sequence homology with and are functionally related to subunits of the bacterial RNA polymerase.

A number of pol II subunits (Rpb5, Rpb6, Rpb8, Rpb10, Rpb12) are shared with RNA polymerase I and RNA polymerase III. Only Rpb4, Rpb7, Rpb9 and the CTD of the Rpb1 subunit are unique to pol II. To understand the transcription mechanisms of pol II in molecular detail, the structure of this complicated machinery has been determined with X-ray chrystallography at atomic resolution. A model of *S. cerevisia*e pol II, lacking Rpb7 and Rpb4 was obtained in 2000 and 2001 (Cramer et al, 2000; Cramer et al, 2001) and revealed the subunit organization of pol II at 2.8 Å resolution.

The two biggest subunits Rpb1 and Rpb2 form the core of the enzyme with smaller pol II subunits being situated on the periphery. The surface of the enzyme is mostly negatively charged apart from the positively charged central "cleft" that is built up by Rpb1 and Rpb2. The pol II active centre is situated at the floor of the cleft. Close to the active site is a protein density, named the "wall" that precludes the straight passage of the nucleic acid through the cleft, forcing it to bend at nearly a right angle. The structure of pol II has also been determined in its elongation state. Several different pol II structures have been determined and these are all very similar, with the only difference being a minor rotation of one side of the "cleft" towards another. This observation implies that the Rpb1 side of the cleft is mobile, capable of adopting different conformations. This mobile part was called the "clamp" and it was suggested to control DNA access to the active site.

Indeed, the crystallization of the elongating form of pol II (in complex with a 3´-tailed DNA template) showed that the clamp swings more than 30 Å over the cleft upon DNA binding, adopting a "closed" conformation in the presence of a DNA-RNA hybrid during active transcription. Further studies of elongating pol II (on assembled *in vitro*

DNA-RNA instead of transcribed), as well as a complex between pol II and TFIIB, shed light on the mechanism of DNA–RNA separation, how translocation is achieved during the elongation phase, how the transcription start site is selected, and details of the nucleotide selection mechanism, which ensures fidelity during transcription. Later, the structure of the complete 12 subunit pol II complex (including the Rpb4 and Rpb7 subunits) has also been resolved. The Rpb4-Rpb7 heterodimer binds to core pol II near the base of the clamp, forcing the clamp to adopt the closed conformation, similar to that seen in the elongating polymerase. The Rpb4/7 dimer was suggested to assist transcription pre-initiation complex assembly, stabilizing TBP-TFIIB bound promoters, and increasing the pol II upstream interaction face of pol II (Bushnell & Kornberg, 2003; Kornberg, 2007).

Recently the crystal structure of pol II isolated from another eukaryote, fission yeast, has been resolved revealing an overall similarity, but also interesting differences in structure between the two yeasts, e.g. in the regions proposed to interact with TFIIB, TFIIF and TFIIH transcription factors (Spåhr et al, 2009)

1.1.1.1 C-terminal domain of RNA polymerase II

The largest subunit Rpb1 has a unique CTD wich has a tandem repetitive structure of characteristic heptapeptide sequence ($Y_1S_2P_3T_4S_5P_6S_7$) with the number of repeats depending on the complexity of the organism and varying from 26 (27) in yeasts to 52 in humans (Allison et al, 1985; Corden et al, 1985a). CTD is crucial for life as has been shown for different organisms. Truncation of CTD by 20 repeats was deleterious to mouse pol II function (Bartolomei et al, 1988). Similarly, in *S. cerevisiae* cells shortening of the CTD to less than eight heptades led to cell death, whereas pol II containing 10 repeats grew essentially as WT albeit with sensitivity to high and low temperatures (Nonet et al, 1987).

The tandem structure of the repetitions also seemed to be important, as strains with insertion of alanines stretches between the repetitive heptads were inviable (Stiller & Cook, 2004; Stiller & Hall, 2002). However, *in vitro* studies of various purified forms of pol II have argued against the absolute necessity of the CTD for transcription, since proteolytic derivative of *Drosophila* (Zehring et al, 1988), budding yeasts (Myers et al, 1998) and human (Dahmus & Kedinger, 1983) pol II lacking CTD were still able to transcribe RNA from some promoters in cell-free systems. This discrepancy in data was the first indication of CTD importance for interaction with other proteins, perhaps

controlling pol II interactions with co-activators during initiation or serving as a docking site for additional accessory proteins in order to assist transcription, rather than itself playing a role in the enzymatic activity of pol II.

Another important feature of the CTD is that almost all amino residues of the repeats can be subjected to various post-translational modifications, such as phosphorylation, glycosylation or proline isomerization. CTD phosphorylation is the most well studied of the post-translational modifications and it has been shown to play a role in different aspects of transcription, including co-activator interactions, nascent mRNA processing, regulation of DNA accessibility etc. Post-translational modifications of CTD came into view when three subspecies of pol II, designated IIA, IIB, and IIO were purified from a number of eukaryotic organisms (Schwartz & Roeder, 1975),(Corden et al, 1985b). Within one organism these forms seemed to differ only by the weight of their largest subunit, which migrated differently in SDS-PAGE analysis. Pol IIB later turned out to represent a product of proteolytic CTD degradation in the course of purification, and this form is thus not physiologically relevant *in vivo*. Pol IIO has been identified as pol II with a hyperphosphorylated CTD, whereas pol IIA is hardly phosphorylated at all. The observed phosphorylation is biologically relevant since substitution of phosphorylated amino acids gives rise to a lethal phenotype (West & Corden, 1995; Yuryev & Corden, 1996). The observation of differently phosphorylated states of pol II stimulated attempts to elucidate the functional differences between these isoforms.

Although 5 potential phosphorylation sites are present in the consensus sequence, Ser2 and Ser5 are the amino residues that are preferentially phosphorylated *in vivo*. Several CTD phosphorylating kinases have been identified to date including the TFIIH component Cdk7, the Mediator component Cdk8, as well as the transcription elongation factor b (p-TEFb, Cdk9) (Cho et al, 2001; Hengartner et al, 1998; Komarnitsky et al, 2000). These kinases are all conserved in evolution and orthologues have been identified in many eukaryotes, including yeast and mammals. The Cdk7 subunit of TFIIH is the major CTD kinase responsible for Ser5 phosphorylation. Immunofluorescence studies investigating the distribution of hyperphosphorylated pol II in Drosophila polythene chromosome revealed pol IIO localization to actively transcribing puffs, suggesting that pol IIO represents the elongating form of the polymerase (Weeks et al, 1993). On the other hand only the non-phosphorylated form of pol II can interact with general transcription factors upon pre-initiation complex formation (LU et al, 1991), whereas phosphorylation of CTD prevents pol II entry into the pre-initiation complex.

The CTD of pol II thus undergoes a cyclic pattern of phosphorylation and dephosphorylation during the course of transcription (Figure 1) (Egloff & Murphy, 2008).

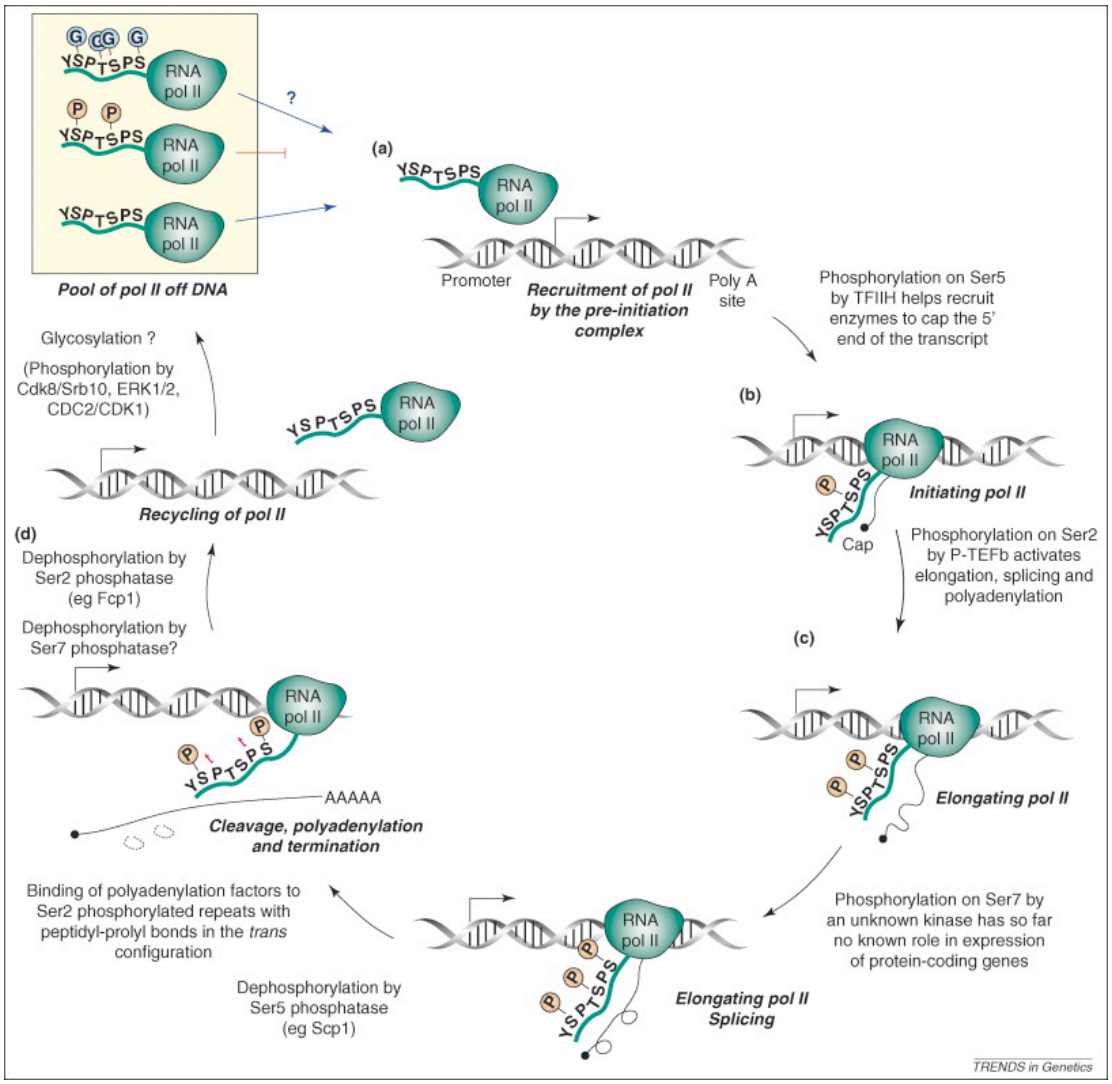

Figure 1. Modification of the pol II CTD heptapeptide during transcription of protein coding genes. Reprinted from Trends Genet, vol. 24 (6), Egloff and Murphy, Cracking the RNA polymerase II CTD code, pp.280-8, Copyright (2008), with permission from Elsevier.

First, non-phosphorylated pol II enters the PIC. After CTD phosphorylation by Cdk7 transcription is initiated. When pol II switches to active elongation, Ser2 is phosphorylated by p-TEFb. CTD phosphorylation plays an important role in the coordination of transcription elongation and mRNA processing. Ser2 phosphorylation is primarily located in the body of the gene and the modification is necessary for the switch to productive elongation. Phospho-Ser5 is reported to serve as a mark for recruiting mRNA capping enzymes. Chromatin immunoprecipitation data shows co-localization of both the Ser5-phosphorylation mark and guanilyltransferase enzymes at the 5′ -end of transcribed genes (Komarnitsky et al, 2000; Morris et al, 2005; Schroeder et al, 2000) and the capping enzymes have been shown to bind directly to CTD

hyperphosphorylated at position Ser5 *in vitro* (McCracken et al, 1997). The recruitment of capping enzymes is dynamic and Ser5-phosphorylation dependent. (Schroeder et al, 2000).

S. pombe p-TEFb (Pch1/spCdk9) interacts with the 5′ capping methyltransferase Pcm1 and forms a near stoichiometric complex. Ser2 phosphorylation as well as p-TEFb recruitment to chromatin is abolished upon Pcm1 depletion, and ChIP analysis shows that Pcm1 and spCdk9 co-localize *in vivo* (Guiguen et al, 2007). Later experiments demonstrated that Cdk7 dependent phosphorylation is necessary and sufficient to promote recruitment of Cdk9-Pcm1 complexes (Viladevall et al, 2009). Moreover, *in vitro* analysis demonstrated that the Cdk7 kinase can "prime" the CTD for Cdk9 dependent phosphorylation. These findings suggest a checkpoints mechanism for transcription elongation, allowing and stimulating Ser2 phosphorylation and transcription elongation only if the mRNA molecule has been properly capped. In addition, in *S. cerevisiae* Cdk7/Kin28 and Ctk1 dependent phosphorylation of the CTD is needed to suppress cryptic transcription at active genes.

Factors involved in mRNA 3′-end processing bind directly to phospho-CTD, in both mammals and yeasts (Ahn et al, 2004; Buratowski, 2005; Hirose & Ohkuma, 2007).

Recently, Ser7 has been identified as an important phosphorylation site on pol II CTD. TFIIH phosphorylates Ser7 and the modification has been linked to processing of pol II transcribed spliceosomal snRNAs in mammalian cells (Akhtar et al, 2009).

Thus, modifications of pol II CTD, including phosphorylation, have complex and multiple functions at all steps of the transcription process (Belotserkovskaya & Reinberg, 2004).

Genome-wide studies in Drosophila (Muse et al, 2007) as well as in human embryonic stem cells (Guenther et al, 2007) have shown that pol II is stalled soon after transcription initiation at nearly 20% of promoters (including heat shock promoters, but also at constitutively expressed genes). Negative elongation factor (NELF) is required for pausing to occur and immunodepletion of this factor leads to a re-distribution of pol II from promoter proximal positions into the bodies of the open reading frames (ORFs). Escape of pol II poised in this way into processive elongation provides an alternative way to regulate mRNA synthesis in metazoan cells in response to environmental stimuli. This switch can be a rate-limiting step of transcription, rather than the transcription initiation reaction itself.

1.1.2 TFIID

TFIID is the transcription factor that starts PIC assembly by binding to DNA. It is a massive multiprotein complex with a total molecular weight of about 750 kDa, consisting of TBP and multiple TBP associated factors (TAFs). TFIID was first isolated by purification of an activity from human cell extracts that was necessary for transcription *in vitro*. One polypeptide of the complex, namely TBP, has been shown to be a major factor responsible for TATA box binding activity of TFIID in yeast, fruit fly, and mammals (Orphanides et al, 1996). TBP can also support transcription initiation on TATA containing promoters in the absence of TAFs, in a cell free transcription system. At least 14 TAFs have been identified in humans and most of them are highly conserved from yeasts to mammals. Thirteen TAF-encoding genes are essential in *S. cerevisiae* (Tora, 2002) and TAFs are not exclusively restricted to TFIID, but can also be part of other coactivators, e.g. SAGA and the SAGA-like complex. TFIID can act as a co-activator complex and human TFIID has been shown to interact with a number of transcriptional activators via its Taf7 subunit, whereas *D. melanogaster* TFIID can bind the Sp1 activation domain. TAF1, the largest subunit of TFIID, has histone acetyltransferase (HAT) activity and might therefore change chromatin structure. Indeed, TFIID, but not TBP alone, is required for pol II transcription from a pre-assembled chromatin template *in vitro* (Wu et al, 1999).

The crystal structure of the TBP/DNA complex has been solved from a number of different species and the molecular details are highly conserved. The TATA box is recognized by the C-terminal part of TBP, which displays 80% identity between yeast and human TBP and is often referred to as core TBP. The N-terminal part of TBP is variable both in length and sequence and is dispensable for DNA binding. This region appears to play a regulatory role, since it can inhibit the binding of core TBP to the TATA box.

The current view on TFIID function is that it binds to core promoters for both TATA-promoters and TATA-less promoters, works as a co-activator for transcription, either by directly recruiting pol II or by modifying either chromatin or other proteins involved in transcription(Thomas & Chiang, 2006).

1.1.3 TFIIB

TFIIB is a single polypeptide transcription factor that performs critical functions in promoter recognition, since it stabilizes the TPB-TATA complex, stimulates pol II

recruitment and directs transcription start site selection. The protein was originally purified from HeLa cells as a factor necessary to tether pol II to TBP-DNA complex (Reinberg & Roeder, 1987). The C-terminal domain of TFIIB is composed of 10 α-helixes forming two imperfect direct repeats of 5 helices each (Tsai & Sigler, 2000). TFIIB core is more resistant to proteolytic degradation and has been shown to bind to the carboxy-terminal domain of TBP (Barberis et al, 1993), and also to interact with DNA both upstream and downstream of the TATA-box (in a sequence independent manner) (Nikolov et al, 1995). However TFIIB can also make sequence dependent DNA contacts in the absence of TBP at the B-recognition elements in the promoter region.

The N-terminal part of TFIIB contains a cysteine rich sequence, forming a Zn ribbon motif. X-ray crystallography studies of TFIIB in complex with pol II have shown that the Zn ribbon motif is involved in interactions with Rpb1 and Rpb2 at the pol II dock domain (Bushnell et al, 2004). This finding is in agreement with the observation that mutations of cysteine residues in the Zn ribbon as well as deletions in the N-terminal part of TFIIB, abolish TFIIB-pol II interactions (Buratowski & Zhou, 1993; Deng & Roberts, 2007). The region of TFIIB, called the B-finger motif, is located immediately after the ribbon domain. A number of mutations in "finger tip" residues have been shown important for transcription start site selection (Zhang et al, 2002). X-ray structure analyses of TFIIB/pol II and pol II/DNA/RNA elongating complexes have revealed that the B-finger projects into the active center of pol II close to the DNA template strand and possibly interferes with nascent RNA.

1.1.4 TFIIF.

Transcription factor IIF (TFIIF) consists of three subunits in budding yeast (Tfg1, Tfg2, and Tfg3) and two subunits (RAP74 and RAP30) in higher eukaryotes (Thomas & Chiang, 2006). The two larger subunits in the yeast complex (Tfg1 and Tfg2) are essential, and homologous to RAP74 and RAP30 respectively (Henry et al, 1994). TFIIF is important for transcription initiation and indispensable for TFIIE and TFIIH recruitment (Orphanides et al, 1996), as well as transcription elongation (Rap74 is required for stimulation of the pol II elongation rate). Rap74 is also involved in pol II recycling, promoting CTD dephosphorylation by the Fcp1 phosphatase (Chambers et al, 1995).

Studies in yeasts have shown that mutations in both *TFG1* and *TFG2* can cause changes in transcription start site utilization. Moreover, *TFG1* mutations functionally interact with mutations in *TFIIB*, *TFG2*, and *RPB9* (Ghazy et al, 2004). Photocrosslinking experiments have mapped the TFIIF-pol II interaction to the Rpb2 lobe and protrusion domains via TFIIF dimerization domain (Chen et al, 2007). This observation implies that the pol II-TFIIB-TFIIF interaction is a determinant for transcription start site selection.

1.1.5 TFIIE

Transcription factor IIE (TFIIE) is composed of two subunits, which in human TFIIE form a tetramer. In *S. pombe*, both the genes encoding TFIIEα and TFIIEβ are essential for viability and a number of functional domains and motifs have been characterized in the two proteins. TFIIE binds to promoter DNA approximately 10 bp upstream of transcription start sites and interacts directly with pol II, TFIIB, and TFIIF (Forget et al, 2004). TFIIE also binds directly to TFIIH and stimulates its kinase activity towards the CTD (Ohkuma & Roeder, 1994). Recent photocrosslinking studies of the initiation complex have mapped TFIIE to the Rpb1 clamp domain, on the opposite side of the cleft from the TFIIF binding site (Chen et al, 2007).

1.1.6 TFIIH

TFIIH is the most complicated of the basal transcription factors and harbors a number of distinct enzymatic activities. TFIIH was initially identified as 9 subunit complex that could be separated into two subcomplexes: the core TFIIH (containing 6 subunits) and the cyclin activated kinase (CAK) subcomplex (also referred as to TFIIK), containing the cyclin H (CycH), cyclin dependent kinase 7 (Cdk7) pair together with the accessory subunit Mat1. Core TFIIH contain two helicases (XPD and XPB) with opposite polarity in their DNA unwinding activities. XPB is a 3′ to 5′ helicase and its helicase activity is needed for both transcription initiation for promoter clearance and DNA repair. XPD is a 5′ to 3′ helicase, which is mainly needed for DNA repair, playing merely a structural role in transcription, being the intermediate subunit that connects core TFIIH with the CAK subcomplex (Thomas & Chiang, 2006).

1.2 CHROMATIN

The mechanism described above for transcription initiation does not reflect the complexity of the *in vivo* situation, since the basal transcription machinery has to act in the context of limited access to DNA within living eukaryotic cells.

1.2.1 Nucleosome

Eukaryotic DNA within the cell has to be tightly packed to fit into nucleus. This is achieved by its organization into a nucleoprotein complex together with architectural proteins called histones, thus forming the so-called chromatin structure. Histone-DNA association not only helps to pack long DNA molecules to fit into the cell, but confers an additional intrinsic level of regulation making underlying DNA less accessible for processes such as DNA transcription, replication, repair etc.

The primary unit of chromatin, the nucleosome core particle, consists of an octamer with two copies of each core histone (H2A, H2B, H3 and H4) with 146 bp of double stranded DNA wrapped around them in approximately 1.7 turns (Figure 2) (Luger et al, 1997). Histones are very conserved basic proteins. Core histones have a globular histone fold domain that is crucial for nucleosome formation and unstructured N-terminal or C-terminal histone "tails", protruding from the nucleosome core, which are important for further packaging into chromatin. The intervening linker DNA found between nucleosome particles can vary in length and makes up the chain of nucleosomes. The "beads on a string" structure can be further compacted into more dense higher order structures. Even packaging into a single nucleosome unit significantly reduces the accessibility of the DNA compared to nucleosome-free naked DNA and hence prevent potential transcription factors from DNA-binding. Nucleosomes, therefore act as general gene repressors. Regulation of transcription must consequently include correct temporal/spatial changes in the chromatin environment in response to external signals.

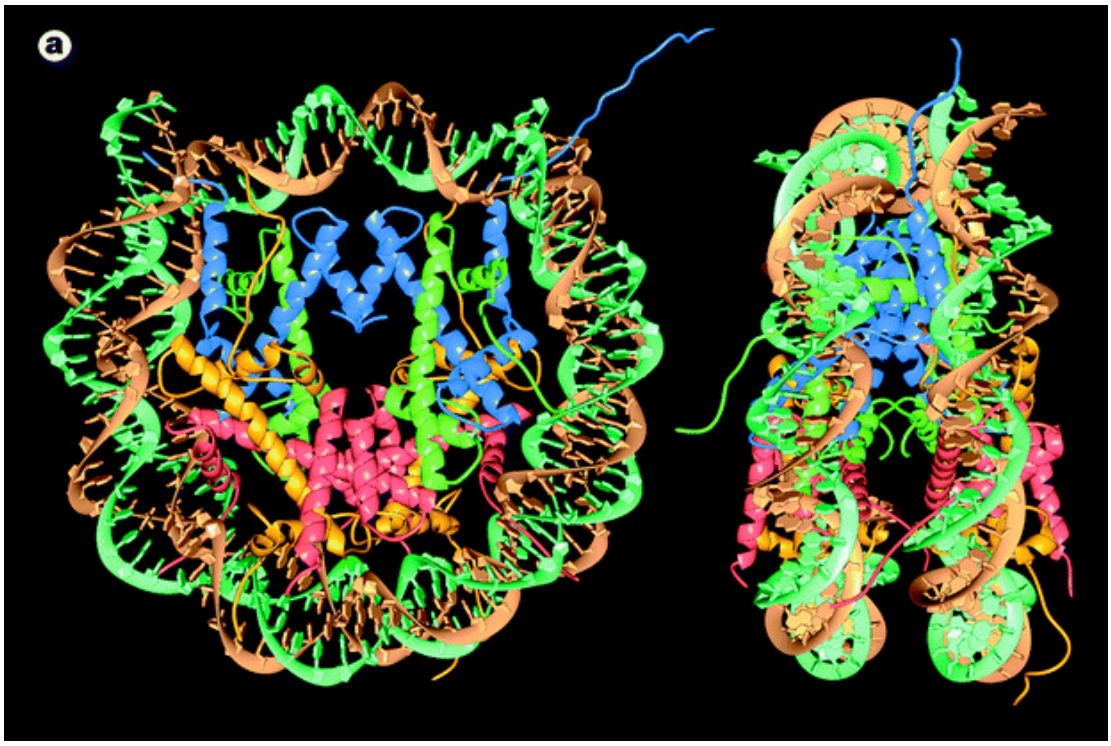

Figure 2. Structure of a nucleosome particle from the front (left) and from the side (right). DNA backbones (brown and turquoise) are wrapped around histone octamer. Histone protein chains are shown: H3-blue; H4-green; H2A-yellow; H2B-red. Reprinted by permission from Macmillian Publishers Ltd: Nature, Luger et al., copyright 1997

1.2.2 Heterochromatin and Euchromatin

Two forms of chromatin are traditionally distinguished. Heterochromatin that is more tightly packed throughout cell cycle is generally considered as non-permissive for transcription and associated with transcriptional silencing. In euchromatin, on the other hand, DNA is more loosely packaged and therefore more accessible for binding by transcription factors and activators, although euchromatic regions are not necessarily actively transcribed at all times. Both the heterochromatin and euchromatin structures are quite dynamic and transiently modulated.

1.2.3 Chromatin remodeling

During the last years the role of chromatin in the regulation of eukaryotic gene transcription has been studied in great detail. Several ways of regulating chromatin structure have been described and these include:

a) ATP dependent chromatin remodeling (sliding or eviction of nucleosomes or parts of nucleosomes by factors utilizing energy from ATP hydrolysis)

b) Use of covalent posttranslational modifications on histone tail residues to change the density of nucleosome packaging. Those modifications are often concerted with ATP dependent chromatin remodeling
c) ATP dependent replacement of the canonical histones with alternative histone variants that alter the stability of a nucleosome and confer specific properties to surrounding chromatin. Variant histones are often targeted to specific positions. H2A.Z is associated with promoter regions and is thought to promote transcription (Cairns, 2009); H2A.Z containing nucleosomes can be less stable than nucleosomes composed of canonical histones and therefore more easily displaced or evicted upon activation of transcription
d) DNA methylation

A large number of chromatin remodeling complexes that act as co-activators and co-repressors have been described and shown to function via diverse mechanisms that can rearrange chromatin structure in response to specific stimuli (Clapier & Cairns, 2009).

1.2.4 Posttranslational histone modifications and histone code

The N-terminal histone tails can be modified at numerous residues (K, R, S). A variety of posttranslational modifications, such as acetylation, methylation, ubiquitination, sumoulation and phosphorylation etc. have been described in the literature (Kouzarides, 2007) (Figure 3). Acetylation and methylation of specific lysines and arginines have attracted the most attention and are best studied. Lysines can be acetylated at position ε-N by histone acetyltransferases (HAT) or methylated by histone methyltransferases (HMT), leading to mono-, di-, or tri-methylation. Acetylation and methylation are reversible and can be removed by histone deacetylases (HDAC) or histone demethylases (HDM). The chemical consequences of covalent modifications differ as the Ac group is negatively charged, which causes a change of the charge of the respective lysine, while transfer of a methyl group does not. In addition, several histone residues in the same nucleosome (not necessarily on the same histone tail) can be modified at the same time creating a particular pattern of modifications. Analysis predominantly of histone methylation and acetylation at ε-N of lysines revealed that particular histone modification could correlate with active or repressed chromatin. Methylation of H3K4 (Santos-Rosa et al, 2002) and general histone acetylation coincide for instance with actively transcribed locations, whereas H3K9, H3K27 and H4K20 methylation are linked to silenced loci (Ruthenburg et al, 2007). Moreover,

genome wide chromatin immunoprecipitation studies have demonstrated that euchromatin assigned H3K4 methylation and heterochromatin localized H3K9 methylation are mutually exclusive (Lachner et al, 2004). These observations led to the hypothesis of a "histone code", suggesting that particular patterns of modification dictate the establishment of a certain chromatin environment. These modifications sometimes can be passed on to the daughter cell, thus generating an epigenetic memory.

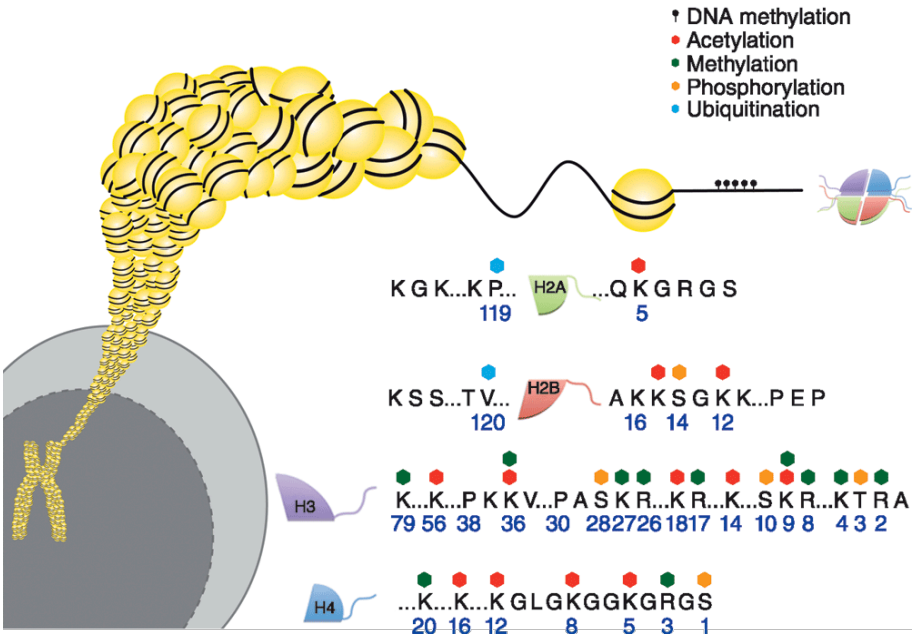

Figure 3. Genome compaction and histone modifications. Reprinted from Essay in Biochemistry. Bou Kheir T. and Lund A.H., Epigenetic dynamics across the cell cycle, Essays Biochem.(2010) **48**, 107-120© the Biochemical Society

Histone modifications have been studied using mainly two approaches. First, the overall distribution of a particular modification can be assessed genome wide using ChIP-chip methodology. Even if this approach has generated a wealth of information about average modification status of the cell population, it does not give much insight about the palette of modifications within a single nucleosome. To address this question mass-spectrometry is nowadays often used (Young et al, 2009).

Histone acetylation often promotes transcription and many transcriptional co-factors and activators possess HAT activities, e.g. GCN5 and p300/CBP. Two different, but not mutually exclusive, models resulted from the analysis of correlations between modification and transcriptional state and explain how histone modifications can affect DNA accessibility. First, reversible histone acetylation (or phosphorylation) can simply modulate a net charge of the nucleosome, thereby altering electrostatic interactions

between histones and negatively charged DNA. Second, histone modifications (both methylation and acetylation) can induce a cascade of events, resulting in chromatin remodeling. The modifications are recognized and bound by specific protein domains, e.g. chromodomains or plant homeodomains (PHD) recognize methylated histones, whereas bromodomains interact with acetylated histone tails. This type of effector proteins that recognize specific histone modifications, in turn promote recruitment of chromatin modifying complexes that shape chromatin and thus assist either gene silencing or activation.

Even if the histone code hypothesis suggests that there is a simple, linear correlation between modifications and transcriptional activity, this is not always the case *in vivo*. Broad correlations observed on the genome wide level can often be difficult to observe at individual genes. In addition, different modifications may functionally interact in complex and unpredictable manners. In murine ES cells the specific pattern of «bivalent» simultaneous H3K4 and H3K27 histone methylation (previously thought not to be coinciding) has been described for the genes related to early differentiation that are normally silent in stem cells (Azuara et al, 2006; Bernstein et al, 2006). Only genes containing solely H3K4 methylation are actively transcribed, while regions associated with bivalent marks are held repressed "primed" or "poised" for subsequent instant activation upon early development. Later it was shown that H3K27 methylation in human (and murine) ES is very often followed by H3K4 methylation and thousands of genes have both marks simultaneously (Mikkelsen et al, 2007; Pan et al, 2007; Zhao et al, 2007). In the course of differentiation either the repressive or activating mark is removed and the gene is induced or remain silent, thus providing yet another possible mechanism of transcriptional control. This feature is not unique to stem cells since bivalent marks were found at some pluripotency-associated transcription factors (NANOG, SOX2) in cells induced for differentiation (Pan et al, 2007).

1.2.5 ATP dependent remodeling

Specific enzymes can utilize energy derived from ATP hydrolysis to alter chromatin structure. These factors can participate in actively sliding (moving histone octamer along DNA), loading, ejecting (displacing histone octamers from DNA), disrupting (e.g. removal of H2A-H2B dimers) or restructuring nucleosomes (e.g. exchanging canonical H2A-H2B dimers with ones containing histone variants) (Cairns, 2005). All the ATP-remodelers, whether they are single subunit enzymes or larger multiprotein

complexes, contain a catalitic subunit that belongs to the Snf2 family of the SF2 superfamily of ATPases (Clapier & Cairns, 2009).

SWI/SNF –the protein that gives the name to the family of ATP-remodelers- was first identified in budding yeasts in screens for genes regulating mating type switching (SWI) and involved in sucrose fermentation (Sucrose Non Fermenting)(Sudarsanam & Winston, 2000)). Later other members of the Snf2 family were identified based on sequence similarity in the ATPase domain. The characteristic feature of Snf2 family proteins is conserved region including 7 helicase-related motifs similar to DExx box helicases, although no intrinsic helicase activity has been reported. Comparison to strand-separating enzymes reveals that Snf2 family proteins lack the motif needed to detach the two strands from one another, so the enzymes are in fact translocases and not helicases (Dürr et al, 2006).

The Snf2 family proteins can be classified into subfamilies in two different ways. A more traditional classification is based on the additional functional domains adjacent to the helicase like domain within the ATPase subunit (Clapier & Cairns, 2009; Ho & Crabtree, 2010; Saha et al, 2006). Depending on conserved sequence motifs flanking the ATPase domain, these ATP-remodelers are divided into the following subfamilies: SWI2/SNF2, ISWI, CHD1 and INO80. The ATPases of the SWI/SNF family contain an N-terminal helicase SANT-like domain together with a C-terminal bromodomain (Horn,Peterson 2001) that can recognize acetylated histones; members of the CHD1 family contain double tandem N terminal chromodomains; ISWI family proteins are characterized by a SANT domain adjacent to a SLIDE domain, both functioning together as a histone-binding module. Accessory motifs within associated subunits often control the activity of the remodeling complexes or help to determine the targeting of these activities to specific genomic locations.

Another way to classify these proteins is based solely on the sequence comparison of conserved helicase like domain in Snf2 family proteins throughout all eukaryotes (Flaus et al, 2006). All ATP remodelers share some common features: apart from DNA affinity, they also possess affinity to nucleosomes, hydrolyze ATP and possess a DNA dependent ATPase activity that breaks DNA-nucleosome contacts. One nucleosome forms 14 contacts with surrounding DNA that have to be broken, not necessarily simultaneously, to move the nucleosome on DNA.

The CHD branch is conserved from *S. cerevisiae* to mammals with one ortholog in budding yeasts, two in fission yeasts and 9 in humans (Jae Yoo et al, 2002; Marfella & Imbalzano, 2007; Woodage et al, 1997).

The signature motifs of CHD remodelers are N-terminal double chromodomains (Woodage et al, 1997), implied to play a role in chromatin targeting. This group is further subdivided into subgroups depending on the presence of additional sequence motifs. Mammalian CHD1 and CHD2, as well as ScChd1, SpHrp1 and SpHrp3 fall into the same subgroup, having an C-terminal DNA binding domain that binds preferentially to A/T rich sequences (Stokes & Perry, 1995). Chd1 proteins are implicated in pol II transcription elongation in a range of eukaryotes. For instance dCHD1 is localized to the highly transcribed regions (puffs) of polytene chromosomes in a pattern highly similar to elongating pol II (Stokes et al, 1996). Both *S. pombe hrp1Δ* and *S. cerevisiae chd1Δ* are deficient in transcription termination (Alén et al, 2002). Human CHD1 binds selectively to H3K4-Me mark via double chromodomains *in vitro* (Sims et al, 2005). Chd1 can play multiple roles in chromatin remodeling. Chd1 proteins have been suggested to play a role in transcription coupled nucleosome disassembly, primarily at the transcription start site (Walfridsson et al, 2007) as well as to act as a chromatin assembly factor both *in vitro* and in *vivo* (Konev et al, 2007; Lusser et al, 2005; Walfridsson et al, 2005).

1.3 MEDIATOR AND ITS ROLE IN TRANSCRIPTION.

1.3.1 Discovery of the Mediator

The over-expression of the Gal4 activator leads to reduced expression of genes, lacking the respective binding site (Gill & Ptashne, 1988), however the sequestering of Gal4 by the co-expression of Gal80, which binds Gal4 activating domain, can partially restore expression. Similarly, expression of the activating domain of the viral transcriptional activator VP-16 causes a significant decrease in expression of unrelated gene. Hormone dependent transcription factors can also affect expression of each other's target genes in HeLa cells–an indication that the amount of transcription factors might be limited (Meyer et al, 1989). This effect of inhibition by unrelated activator suggested that different activators might compete for a particular intermediary factor that conveys their enhancer function on transcription and is common for various activators. Furthermore, *in vitro* transcription experiments using partially purified yeast proteins have shown that polymerase II assisted by basal transcription factors could produce RNA from naked (not nucleosomal) DNA template but was unable to respond to the addition of an activator (Flanagan et al, 1991; Kelleher et al, 1990). Together, these

observations suggested the existence of a co-activator complex that could interact with activators and stimulate the activity of the general transcription machinery.

Attempts to isolate the missing component that could restore the ability to stimulate activator-dependent transcription *in vitro* led to the discovery of a large protein complex named Mediator (Kim et al, 1994). Mediator thus is a multiprotein complex that can convey information from activators to basal transcription machinery.

Mediator has been purified as a holoenzyme with RNA polymerase II and TFIIF (Kim et al, 1994). It is required for transcription of virtually all pol II dependent genes since the mutation of the essential Mediator subunit and mutation of the largest pol II subunit Rpb1 had similar consequences for gene expression (Holstege et al, 1998).

Some of budding yeast Mediator subunits were independently identified in genetic screens for suppressors of Ty insertion in *S. cerevisiae* (Thompson et al, 1993; Yamaguchi et al, 2001), as well as for suppressors of cold sensitivity caused by pol II CTD truncation (Nonet & Young, 1989),(Koleske et al, 1992), which implied their functional relation to the CTD and their importance for RNA polymerase II transcription. All but four Suppressors of RNA polymerase B mutations (Srb) proteins were identified as subunits of transcriptionally active Mediator complex.

The four subunits Srb8, 9, 10 and 11 were isolated as a separate complex in budding yeasts (Borggrefe et al, 2002). Nevertheless, the results of Hengartner (Hengartner et al, 1995) suggested that Srb8-11 could associate with Mediator and other components of the pol II transcription machinery *in vivo*.

Mediator components were also identified as positive transcriptional regulators (Carlson, 1997). A Gal11/Med15 mutation reduced transcription of enzymes involved in galactose catabolism (encoded by Gal1, 7, 10) to at least 30% of the wild type level upon galactose induction. The effect was not connected to diminished amounts of Gal4, suggesting that Gal11/Med15 was required for mediating the *in vivo* response to the Gal4 transcription factor (Suzuki et al, 1988).

Several similar Mediator-like co-activator complexes were purified from human and murine cells in different labs, based either on their affinity to known transcriptional activators (factors) (Fondell et al, 1996; Näär et al, 1999; Rachez et al, 1998), or to CTD (Jiang et al, 1998; Näär et al, 1999), their activity in *in vitro* transcription (Kretzschmar et al, 1994), or based on homology to known yeast Mediator components.

TRAP/SMCC (Gu et al, 1999; Ito et al, 1999), DRIP (Rachez et al, 1998), ARC-L, CRSP (Ryu & Tjian, 1999; Ryu et al, 1999), NAT (Sun et al, 1998), PC2 (Kretzschmar et al, 1994)- all these complexes turned out to represent metazoan Mediators.
Mammalian Mediator–like complexes include large 1-2 MDa complexes such as TRAP/SMCC, ARC-L, mouse and rat Mediators as well as smaller, around 500-700 kDa complexes including CRSP, Positive Cofactor PC2. The composition of those complexes varied significantly and only a subset of polypeptides was found in nearly all Mediator preparations. The variety of subunit composition could reflect the existence of distinct Mediator assemblies with varying transcriptional behavior.

The primary difference in the composition between Small(S-) and Large(L-) Mediator complexes was the absence or presence of a four subunit kinase subcomplex composed of Med13/Trap240, Med12/Trap230, Cdk8 and Cyclin C (Paper I). Systematic analysis using Multidimentional protein identification technology (MudPIT) by Sato et al. addressed the subunit composition of Mammalian Mediator-like complexes. 30 Mediator components previously reported by others were identified in this study along with two new polypeptides previously not reported as components of Mediator like complexes (Sato et al, 2004).

Primary sequence conservation between yeast and mammal Mediator subunits is not obvious for the majority of subunits. Only eight Mediator subunits were at first acknowledged to be evolutionary conserved in eukaryotes (Malik & Roeder, 2000) and it was initially questioned if Mediator really was a evolutionary conserved entity. Intensive cross-species sequence comparisons studies from different labs in combination with biochemical observation allowed detection of metazoan homologs for nearly all yeast Mediator subunits, therefore unified nomenclature for Mediator subunuts was proposed (Table1) (Bourbon et al, 2004).

1.3.2 Modular structure of Mediator complex

The first view on the Mediator was given by electron microscopy single particle analysis of *S. cerevisiae* Mediator at low resolution (40Å) (Asturias et al, 1999). It revealed that purified Mediator has a compact globular structure, but in the presence of pol II a fraction of Mediator can bind to the enzyme and adopt a fully extended conformation “embracing” globular polymerase and forming a holoenzyme. In this

conformation three different domains of Mediator could be distinguished on the image. The biggest module was denoted the head domain, followed by the middle and the tail domains. Interestingly, Mediator was unable to form a stable association with CTD-less pol II, suggesting that this structure is required for holoenzyme formation. Pol II appeared to form multiple contacts with Mediator via its head and middle domain, while the tail domain remained free in solution (Asturias et al, 1999; Dotson et al, 2000).

Early biochemical analysis of the *S. cerevisiae* Mediator-complex also implied a modular organization of Mediator. Urea dissociation analysis of immobilized purified Mediator (Lee & Kim, 1998), analysis of subunit composition of Mediators from deletion strains for known subunits (Myers et al, 1999), *in vitro* reconstitution combined with immunoprecipitation experiments (Kang et al, 2001), as well as two hybrid-screen data (Guglielmi et al, 2004) enabled a detailed characterization of the polypeptides organization within the complex and suggested that it was constructed from smaller subcomplexes. All these data combined with transcriptional profile analysis in deletion strains for non-essential subunits in *S. cerevisiae* supported an organization of subunits into specific subcomplexes (van de Peppel et al, 2005).
Nowadays the subunit organization for at least budding yeasts Mediator is well established.

1.3.2.1 The head module

The head module is critical for Mediator function. First, the Med17 subunit is crucial for expression of almost all pol II transcribed genes. Inactivation of the head module leads to impaired recruitment of Mediator to at least some promoters (Takagi et al, 2006) and most head module subunits are conserved in higher eukaryotes (Bourbon et al, 2004). EM pictures of the pol II- holoenzyme revealed that the head module formed extensive contacts with pol II (Dotson et al, 2000). All but 2 subunits of head module (Med18 and Med20) are essential for cell viability.

Using the baculovirus expression system, it was possible to co-express and reconstitute a full 7 subunits head module. It was composed of the Med6, 8, 11, 17, 18, 20 and 22 subunits (Takagi et al, 2006). Further EM single particle analysis revealed that the head module was organized around a three subunit compact core, comprised of Med11, 17, and 22 (Cai et al, 2010). Med6 and Med8 subunits form the proximal end of the head module structure serving as an interacting surface with the rest of Mediator. Med6 is suggested to bridge the middle and core modules, by directly interacting with Med21.

Table 1. Mediator complex subunits.

	S. cerevisiae	Module	*S. pombe*	*H. sapiens*	Essential
Med1	Med1	M	Pmc2	TRAP220, CRSP200 ARC/DRIP205	N
Med2	Med2	T			N
Med3	Pgd1/Hrs1	T			N
Med4	Med4	M	Pmc4/Med4	TRAP/DRIP36	Y
Med5	Nut1	T			N
Med6	Med6	H	Pmc5	hMed6, DRIP33	Y
Med7	Med7	M	Med7	hMed7, DRIP34, CRSP33	Y
Med8		H	Med8/Sep15	ARC32	Y
Med9	Cse2/Med9	M		Med25	N
Med10	Nut2/Med10	M	Nut2	hNut2, hMed10	Y
Med11	Med11	H		HSPC296	Y
Med12	Srb8	K	Srb8	TRAP230, ARC/DRIP240	N
Med12L				TRALPUSH	
Med13	Ssn2/Srb9	K	Trap240	TRAP240, ARC/DRIP250	N
Med13L				PROSIT240	
Med14	Rgr1	M/T	Pmc1	TRAP170, ARC/DRIP150	Y
Med15	Gal11/Spt13	T	Med15	ARC105, PCQAP	N
Med16	Sin4	T		TRAP95, DRIP92	N
Med17	Srb4	H	Srb4	TRAP80, ARC/DRIP77	Y
Med18	Srb5	H	Pmc6/Sep11	P28b	N
Med19	Rox3	H	Rox3	LCMR1	N
Med20	Srb2	H	Med20	hTRFP	N
Med21	Srb7	M	Srb7	hSrb7	Y
Med22	Srb6	H	Srb6	Surf5	Y
Med23				ARC/DRIP130, CRSP130, hSur2	
Med24				ARC/DRIP100, CRSP100	
Med25				ARC92	
Med26				ARC70,CRSP70	
Med27			Pmc3	TRAP37,CRSP34	N
Med28				Fksg20	
Med29				Hintersex	
Med30				TRAP25	
Med31	Soh1	M	Soh1/Sep10	hSoh1	N
CDK8	Srb10/Ssn3	K	Srb10	CDK8	N
CDK8L				Cdk11/CDK8L	
CycC	Srb11/Ssn8	K	Srb11	CycC	N

The Med6-Med21 interaction is essential *in vivo* and deletion of N-terminal part of Med21 involved in this interaction is lethal.

Med18 and Med20 are situated on the periphery of the head-middle binding surface and are connected to the Med11/17/22 triad via the Med 8 subunit (Cai et al, 2010). Med18 and Med20 form a functional subcomplex and have a similar effect on global gene transcription patterns. Deletion of the Med8 C-terminal domain leads to loss of the Med18/Med20 subunits from the purified Mediator and impaired transcriptional activity in nuclear extract (Larivière et al, 2008). The Med18/20/8 triad is crucial for Mediator interactions with pol II. Genetic studies in *S. pombe* demonstrated that the Med8 C-terminal domain is needed for Mediator-pol II interaction (Mehta et al, 2009) and recent EM studies revealed that the Med18 and Med20 subunits mediate contacts with pol II via the Rpb4/Rpb7 dimer (Cai et al, 2009; Cai et al, 2010). This interaction is crucial since deletion of either Med18 or Med20 in a combination with an rpb4 deletion causes synthetic lethality.

Med19, previously reported to be a component of the head module is, instead required for connecting the head module to the middle module (Baidoobonso et al, 2007). The head module subunits also interact with general transcription factors. The head module seems to form multiple contacts with TBP (Cai et al, 2010), interact with TFIIH via its Rad3 subunit (Esnault et al, 2008), as well as with transcription factor TFIIF (Takagi et al, 2006).

1.3.2.2 The middle module

The middle module is composed of 7 subunits Med 1, 4, 7, 9, 10, 21, and 31. X-ray crystallography data revealed that the highly conserved Med7 and Med21 subunits form a flexible hinge that can undergo repositioning of about 10Å. Additionally the head module subunit Med6 makes a flexible connection between Med17 of the head module and the Med7-Med21 heterodimer of the middle module, interacting with both Med21 and Med7 (Baumli et al, 2005). The structural flexibility might explain the change in structure between closed and opened conformation observed upon Mediator binding to pol II. Med7 is an architectural subunit of the middle domain that is stably associated with Med21 via C-terminal domain and Med31 via N-terminal domain (Koschubs et al, 2010).

Further, Med4, Med9 and Med10 are associated with Med7/ Med21 / Med31 subcomplex. Med4 interacts with the middle region of Med7 and with Med9. Med1 and Med10 both bridge between Med7/Med21 and Med4/Med9. Med10 N-terminus is

exposed and interacts with Med 14 subunit that connects the tail and middle modules (Li et al, 1995).

1.3.2.3 The tail module

The tail module of the Mediator complex was at first suggested to be specific to *S. cerevisiae* Mediator. It consists of the Med 2, 3, 5, 14, 15 and 16 subunits (Béve et al, 2005; Guglielmi et al, 2004; Li et al, 1995). The N-terminal portion of the Med14 subunit interacts with the N-terminal portion of Med10, thus connecting the tail to the middle module of *S. cerevisiae* Mediator (Koschubs et al, 2010). This module is less conserved and only half of the six subunits (Med14, Med16 and Med15) have clear metazoan homologs (Bourbon et al, 2004). It was also suggested that Med16 plays a structural role since Med16 deletion leads to loss the Med2, Med5, Med15/Gal11 and Med3/Pgd1 subunits (Béve et al, 2005; Myers et al, 1999; Zhang et al, 2004).

1.3.2.4 The kinase module

The kinase module consists of the Cdk8 kinase, Cyclin C, Med12, and Med13. The kinase module was not identified as a part of transcriptionally active budding yeast Mediator (Borggrefe et al, 2002) but has been identified in Mediator isolated from higher eukaryotes. The kinase module has been suggested to have repressive function, which has been coupled to kinase activity of Cdk8 subunit. First, it was suggested that an inhibitory premature phosphorylation of pol II CTD by Cdk8 kinase could prevent pol II from entering PIC and thus inhibit transcription initiation (Hengartner et al, 1998). Second, metazoan Cdk8 could phosphorylate the TFIIH transcription factor, thereby inhibiting its ability to phosphorylate pol II CTD and support transcription, whereas small (S-) Mediator lacking Cdk8 kinase had a stimulatory effect on transcription (Akoulitchev et al, 2000).

Data from mammalian systems also demonstrated that Mediator like complexes can inhibit transcription depending on their association with the kinase module. The mammalian ARC-L kinase containing complex and the small CRSP complexes not only differ in structure, but also behave differently in transcription assays. In contrast to CRSP Mediator, ARC-L could not support activated transcription *in vitro* (Taatjes et al, 2002). Interestingly, the kinase associated inhibitory form of Mediator was found to be the most common *in vivo* (Malik & Roeder, 2005). EM studies of *S. pombe* S- and L-Mediator complexes from our group demonstrated that pol II and the Cdk8 module might compete for the same binding site, therefore the kinase module might suppress

transcription independently of the Cdk8 enzymatic activity, simply by occluding pol II from binding (Elmlund et al, 2006) papers I-III).

1.3.3 Mediator Recruitment- implications for activation and repression

The initial idea that Mediator functions simply as a communicator between activators and the basal transcription machinery was supported by the identification of many putative targets for transcriptional activators within the Mediator complex. First, the Mediators prepared from the deletion strains lacking the tail subunits Med2, Med3, and Med15 were defective in activation of transcription but could still stimulate basal transcription (Lee et al, 1999). Second, Mediator subunits were shown to directly bind transcriptional activators. These interactions, even though mainly assigned to tail subunits (Gal11-Gcn4 (Herbig et al, 2010; Jedidi et al, 2010; Lee et al, 1999), Gal11-Gal4 (Jeong et al, 2001), Gal11-Swi5 (Bhoite et al, 2001), Gal11-Pdr1 (Thakur et al, 2008), Gal11–Oaf1 (Thakur et al, 2009)), were also reported for the core Mediator subunit Med17 and Gal4 (Koh et al, 1998).

Low-resolution pictures demonstrated that mammalian Mediators use different interfaces for binding to specific activators (VP-16 and SREBP-1) and that binding could induce distinct conformational changes (Taatjes et al, 2004a; Taatjes et al, 2002; Taatjes et al, 2004b), giving insight into how a single complex might concert signals from a variety of activators. Indeed, in mammalian Mediators particular subunits were demonstrated to be targets of transcriptional activators suggesting a mechanism for cell specific transcription (Blazek et al, 2005).

The existence of various forms of Mediator suggests a simple model that both activation and repression can be achieved by recruitment of either transcriptionally active or inactive Mediators to promoters upon binding of particular transcription factors.

Mo et al. investigated Mediator interactions with the human transcription factor CCAT/enhancer binding protein beta (C/EBPβ). C/EBPβ activity is regulated by RAS dependent phosphorylation that induces conformational change, turning C/EBPβ from repressor to activator. They demonstrated that phosphorylated C/EBPβ could interact with S-Mediator and recruit it to target promoter whereas the non-phosphorylated form of C/EBPβ recruited L-Mediator (Mo et al, 2004). This finding therefore suggests that the recruitment model is valid at least at some promoters. In agreement with this idea,

the *S. cerevisiae* repressor Tup1 has been shown to bind Cdk8 and CycC (Zaman et al, 2001).

Contradicting this model, genome-wide chromatin immunoprecipitation data demonstrated very similar patterns for the kinase module and core Mediator binding. Both the kinase module and core module subunits are bound to IGRs and ORFs of transcribed as well as silenced genes (Andrau et al, 2006; Zhu et al, 2006).

Biochemical data from human systems demonstrated that the kinase module acts as a molecular switch between active and non-active forms effectively suppressing transcription independently of the kinase activity (Knuesel et al, 2009). These data are therefore very similar to the model previously presented for the kinase module in *S. pombe* Mediator (Elmlund et al, 2006). Together these observations suggest that core Mediator and the Cdk8 module might be pre-recruited (together or separately) to promoter regions making up the platform for rapid gene activation (or repression in case of highly transcribed genes). In response to signaling events they would undergo rearrangement, promoting the dictated outcome on gene expression level. The regulation might be achieved via activator recruitment that might prompt Cdk8 dissociation from Mediator.

1.3.4 Mediator: multiple roles in transcription

Studies of metazoan Mediators have established that the co-activator function of Mediator is much more complicated than it was initially suggested and acting through different mechanisms. Moreover, Mediator is regulated by many different gene specific transcription factors and the complex is a target of many intracellular signaling pathways (Casamassimi & Napoli, 2007).

Recent studies demonstrated that Mediator could control gene expression affecting chromatin regulation. The Mediator subunit Med12 could selectively assist negative transcription regulator REST in gene silencing at target promoters by affecting chromatin structure. REST forms a ternary complex with Med12 and the H3K9 methyltransferase G9a. Recruitment of G9a and inhibitory H3K9 methylation is dependent on Med12. Indeed, Med12 knockdown alleviates REST directed repression at repressor element 1(RE1) sites (Ding et al, 2008).

Another example of Mediator promoting chromatin alteration came from biochemical analyses of human L-Mediator. Meyer et al. have demonstrated that Cdk8 containing L-Mediator can interact with GCN5L histone acetyltransferase and cooperatively

phosphoacetylate Ser10/Lys14 on histone 3 *in vitro*. Moreover, knockdown of Cdk8 leads to reduction of *in vivo* histone phosphoacetylation by 80%. GCN5L associated L-Mediator still had a repressive function *in vitro* (Meyer et al, 2008).

A number of reports have indicated a role for Mediator in transcription elongation. Cell-free transcription experiments in HeLa cells in non-fractionated extracts demonstrated that the transcription elongation factor Spt4-Spt5 was required for optimal Mediator function (Malik et al, 2007). Another indication that Mediator could control a post recruitment step came from studies of serum-activated genes in tumor cell lines. ChIP demonstrated that the Cdk8 kinase might promote transcription at immediate-early genes upon serum stimulation. Depletion of Cdk8 led to slower elongation and impaired pol II CTD phosphorylation in the body of the gene upon serum stimulation, whereas pol II occupancy at the targets was generally unaffected. Only L-Mediator and not S-Mediator was shown to directly interact with p-TEFb and recruitment of this elongation factor was impaired in Cdk8 knockdowns (Donner et al, 2010).

Med12 was suggested to regulate embryonic cell pluripotency by regulating expression of Nanog dependent genes including Nanog itself in murine ES cells. Microarray expression analysis demonstrated a significant overlap of Med12 and Nanog target genes. Moreover Med12 and Nanog were enriched at Nanog target promoters under proliferation conditions but dissociated upon differentiation (Tutter et al, 2009).

Recent findings suggested the mechanism for such regulation. Kagey et al. demonstrated that Mediator could directly cooperate with the cohesin complex, which promotes DNA loop formation between enhancer and core promoter at highly expressed pluripotency genes in murine embryonic stem (ES) cells (Kagey et al, 2010). A screen for factors needed for maintenance of Oct4 transcription factor expression, along with genes coding for known pluripotency transcription factors (Sox2, Oct4, Nanog), also identified genes encoding Mediator complex subunits and unexpectedly Cohesin complex components. The Cohesin complex is best known for its role in mediating sister chromatid cohesion.

Cohesin subunit Smc1a, cohesin loading factor Nipbl, and Mediator subunit Med12 were shown to interact with each other and bring together the enhancer and the core promoter regions at Oct4 and Nanog transcription factor genes, thus forming and stabilizing enhancer-promoter DNA loops. Interestingly these loops were observed

only at actively transcribed genes and reduction of Med12 or Smc1a expression led to diminished enhancer-core promoter interaction.

It appears now that Mediator has multiple functions in transcription regulation and function as a signal-processor centre for various genetic programs. Apart from the long ago established role in RNA polymerase II recruitment upon pre-initiation complex assembly, Mediator can also affect transcription at post-recruitment step, contribute to chromatin remodeling and thereby promote silencing or transcription depending on the context (Malik & Roeder, 2010).

1.4 *S. POMBE* AS A MODEL ORGANISM

S. pombe is a useful model system to study eukaryotes for several reasons. It has relatively small genome, which was fully sequenced already in 2001 and annotated in 2002 (Wood et al, 2002). *S. cerevisiae* has been more frequently used as a model system for eukaryotic transcription and *S. cerevisiae* genes are often better annotated compared to *S. pombe*. On the other hand, *S. pombe* genome does not seem to show evidence of large genome duplication, leading to low redundancy level of the genes and simplifying the characterization of protein function (Bähler & Wood, 2004). *S. pombe* has been particularly valuable in studies of molecular basis of cell cycle control (Nurse, 2002). Large evolutionary divergence between fission and budding yeasts makes *S. pombe* an extremely helpful model organism alongside with *S. cerevisiae* since comparisons between these species can be very useful for extrapolation to other eukaryotes.

Moreover fission yeasts in some aspects are more mammalian-like than budding yeasts, e.g. in transcription initiation site position (Choi et al, 2002). Many molecular biology techniques developed for *S. cerevisiae* are generally applicable in *S. pombe.* Yeasts in general are easy to handle, relatively inexpensive and propagate quickly making it is easy to obtain a large amount of material in a short time for e.g. biochemical studies.

2 RESULTS

Mediator complex was first isolated from budding yeasts (Flanagan et al, 1991; Kelleher et al, 1990; Kim et al, 1994). Identification of metazoan Mediator-like complexes with similar function in transcription led to the conclusion that the function of Mediator could be evolutionary conserved. However the low primary sequence conservation for the majority of Mediator subunits between fungi and metazoan co-activator complexes seemed to argue against this conclusion and raised the possibility that the subunit composition and also molecular function of Mediator might be different between yeast and higher eukaryotes.

At the start of our project, a number of mammalian Mediator-like complexes had been described. They could be divided into two large groups, the larger L-Mediators that contained a number of additional subunits and also a the conserved CyclinC-CDK8 kinase pair and the smaller S- or C-Mediator, which lacked CyclinC-CDK8 and many other Mediator components, and instead contained one additional component, CRSP70/ Med26.

S. cerevisiae Mediator could be isolated either in free form or in complex with pol II, forming a holoenzyme (Myers et al, 1998). Cyclin C and Cdk8 were not present in the purified Mediator, but instead purified in complex with two additional yeast proteins, Srb8 and Srb9, which lacked clear counterparts in the mammalian Mediator complex.

When we initiated our work, it was also not clear how conserved the Mediator subunits were. Mediator had only been purified from a limited number of species and it was therefore difficult to make sequence comparisons. At the time, only eight of the 25-30 Mediator components were acknowledged to be conserved from yeasts to humans (Bourbon et al, 2004; Malik & Roeder, 2000).

Fission yeast Mediator had previously been purified as a holoenzyme by conventional chromatography in association with pol II (Spåhr et al, 2001). The fission yeast Mediator was significantly smaller then *S. cerevisiae* or mammalian Mediator-like complexes and only contained 13 subunits, possibly due to partial protein degradation in course of purification or loss of loosely associated subunits. We wished to better characterize the *S. pombe* Mediator complex and to address the question of whether any alternative forms of Mediator existed, similarly to the situation in higher eukaryotes.

2.1.1 Paper I

We decided to take advantage of Tandem Affinity Purification (TAP) method in combination with optimized whole cell extract preparation in order to diminish proteolysis.

We introduced a TAP-tag on the core subunit Med7 and isolated a Mediator complex, containing two additional high molecular weight subunits homologous to *S. cerevisiae* Srb8 and human TRAR240 respectively according to BLAST sequence similarity searches. Interestingly, the newly identified polypeptides, as well as pol II subunits, were present in substoichiometric amounts to the core Mediator component Med14/Rgr1. We assumed that our preparation might be a mixture of various forms of Mediator. Affinity purification of spTrap240-TAP cell extract allowed us to isolate the alternative form of fission yeast Mediator near to homogeneity. In addition to spTrap240 and spSrb8, it also contained the two smaller subunits homologous to budding yeast Cyclin Dependent Kinase Srb10 and Cyclin Srb11. We assumed that the four indicated subunits might form a submodule. In support of this notion spSrb8 and spTrap240 had similar effect on gene expression, repressing the same subset of genes.

Surprisingly, in contrast to the holoenzyme form of Mediator, no components of pol II were identified in the L-Mediator preparation. We thus demonstrated that fission yeast Mediator can exist in at least two different forms. The larger form does not interact with pol II in contrast to the smaller one that forms holoenzyme with pol II.

We proposed that the four subunit kinase subcomplex formed an evolutionary conserved Srb8-11 kinase module with spTrap240 and spTrap230 being bona fide homologs of Srb9 and Srb8. Our findings therefore provided a simplified model for Mediator organization and helped to connect findings in mammalian cells with observations in yeast.

2.1.2 Paper II

Using the TAP-method we purified the Mediator-pol II holoenzyme from the *Δmed13/trap240*. This Mediator also lacked the Cdk8 kinase activity, so we assumed that Med13/spTrap240 is a subunit required for anchoring the kinase module to the core Mediator. We did not address the subunit organization in this paper (see paper III for details).

Having established a reliable protocol for isolation of Mediator to homogeneity, we wished to test its function as co-activator in a reconstituted *in vitro* transcription

system. We decided to create an *S. pombe* cell-free transcription system from highly purified general transcription factors. Depending on the complexity of a particular factor we employed different strategies for protein purification and expression. The large multisubunit complexes, such as pol II, five-subunit core-TFIIH and the TFIIH-kinase module (TFIIK) were isolated from whole yeast cell extract. Pol II was immunoprecipitated using antibodies directed against pol II CTD. The submodules of TFIIH were isolated using TAP-purification.

The His-tagged recombinant single subunit TFIIB factor was overexpressed in *E.coli*. The His-tagged recombinant two-subunit TFIIE and three-subunit TFIIF were over-expressed in insect cells by co-expression of baculoviruses expressing the respective subunits. All recombinant factors were Ni-affinity purified and then subjected to ion-exchange chromatography.

We reconstituted an *in vitro* transcription system of *S. pombe* GTF together with *S. cerevisiae* TBP from nucleosome-free adh1-promoter containing template. We compared the effects of the holoenzyme and kinase-containing Mediator on basal transcription.

The Mediator holoenzyme stimulated basal transcription in our reconstituted in vitro transcription system, whereas the L-Mediator effectively suppressed transcription in a dose dependent manner.

2.1.3 Paper III

We earlier demonstrated that deletion of Med13 led to loss of Cdk8-kinase from Mediator. EM structural analysis of the C- and L-Mediator states have implied that inability of L-Mediator to interact with pol II is caused by sterical hindrances rather than enzymatic activities of the kinase module (Elmlund et al, 2006). Cdk8 module presence is sufficient to block pol II binding. EM data suggest that the Cdk8 module forms a molecular lid that might cover the pol II binding interface.

Now we decided to further systematically investigate the subunit architecture within the kinase module of L-Mediator. We generated deletion strains for the kinase module subunits in the Med7-TAP and Med13-TAP backgrounds and analyzed subunit composition of isolated Mediators by immunoblotting. The TAP-tag has a significant size and it could potentially disturb interactions within the complex, being a possible cause for pol II dissociation. To rule out this possibility we also included in our analysis preparations from strains bearing TAP-tag on CyclinC/Srb11.

We found that Med13 was indeed the subunit anchoring the kinase module to the core Mediator since Med12, Cdk8 and CycC were lost from the *Δmed13* holoenzyme. The kinase activity or physical presence of Cdk8 was not required for L-Mediator formation. Instead stability of Med12 association was dependent on Cdk8 presence since deletion of Cdk8 caused significant loss of Med12. The knock-out of the Cdk-cyclin pair had a similar effect on Med12 association with C-Mediator. Interestingly, CycC remained associated with Mediator in *Δcdk8* Mediator preparations however its amount was reduced. We demonstrated that the deletion of different subunits of the kinase module has different consequences for architecture of the Cdk8 module, thus suggesting step-wise structural organization of subunits in the kinase module.

In agreement with our previous assumptions the sterical hindrance and not Cdk8 kinase activity seemed to be a predominant factor preventing pol II–Mediator association. Med 13 seemed to present the major obstacle for pol II binding given that Cdk8, CycC and probably also Med12 were not absolutely required to block pol II interactions.

The different behavior of the C- and L-Mediator in transcription, as well as data arguing for the repressive function of the kinase module in both budding and fission yeasts (paper I), induced the question of how regulation of activation and repression might be achieved.

Earlier reports from the human system speculated that only small CRSP, but not the large ARC-L complex could interact with the CTD of pol II, since only small Mediator could be pulled down by recombinant CTD from HeLa nuclear extract (Näär et al, 2002).

Using our highly homogenous Mediator preparations in a CTD binding assay we could demonstrate that S-Mediator indeed binds the CTD (not shown). Surprisingly, we detected the core Mediator subunits in the bound fraction from L-Mediator as well. By immunoprecipitation experiments we demonstrated that an excess of CTD could effectively displace the kinase submodule from L-Mediator and bind S-Mediator. The number of CTD repeats as well as the phosphorylation state of the CTD was of importance. Only non-phosphorylated CTD longer than 11 repeats could effectively displace the kinase module from the rest of Mediator. The phosphorylation of CTD completely abolished its ability to bind Mediator.

It is feasible that interaction with activators or repressors might play a role in transition between a transcriptionally inactive and active states of Mediator.

2.1.4 Paper IV

We focused on characterization of the putative component of fission yeast Mediator Med15. Med15 is a conserved component of the Mediator in many species, but so far we were not able to identify it as a component of fission yeast Mediator despite the fact that the *S. pombe* genome encodes a Med15 orthologue, presumably because of degradation issues. We purified L-Mediator from the yeast extract, prepared under conditions that diminish proteolysis. A polypeptide around 140 kDa was identified as *S. pombe* Med15. Judging from the relative intensities of the bands on the Coomassie stained gel we estimated Med15 to be present in 10-15% of all L-Mediator complexes. We also introduced the tandem C-Terminal FLAG-tag on Med15 and purified Med15 by immunoprecipitaion. We found Med15-FLAG associated with the single polypeptide identified as Hrp1, a CHD1 ATP-dependent chromatin-remodeling protein. Interestingly, other Mediator components were absent in the Med15-FLAG preparations, suggesting that Med15 is not a stable component of the Mediator complex. Co-immunoprecipitation using anti-Hrp1 antibodies confirmed Med15-Hrp1 association. Previously we were not able to identify Med15 in our holoenzyme preparation. We directed antibodies against Med15 and analyzed pol II holoenzyme- and L-Mediator preparations by immunoblotting. We found that both Med15 and Hrp1 are present in L-Mediator but are absent from core Mediator. We concluded that Med15 and Hrp1 might form a subcomplex and that the two proteins are transient components of the L-Mediator complex. We next explored if the physical interaction might also reflect a functional interaction between Med15 and Hrp1. To do so we investigated genome wide transcription profiles in *Δmed15* or *Δhrp1* mutants using the Affimetrix microarray platform. Med15 deletion had larger effects on gene expression, but no fewer than half of the genes that are up-regulated in *Δhrp1* were also up-regulated in *Δmed15*.

To find *in vivo* targets for Med15 and Hrp1 binding we performed genome-wide ChIP-chip analysis. In this study we used *S. pombe* spotted arrays that contained PCR products corresponding to 500 base pairs fragments upstream from the translation start site (InterGenicRegions) and the last 500 base pairs of the coding regions (ORFs). Each array contained 4.960 IGR and 4.976 ORF fragments spotted in duplicates.

A significant overlap in binding targets for Med15 and Hrp1 in IGRs (p=6,55X10^{-81}) as well as coding regions (p=1,22X10^{-14}) suggested that the two proteins might act at the same genomic locations.

We had earlier established a role for Hrp1 in nucleosome disassembly (Walfridsson et al, 2007). Comparison of Med15 and Hrp1 binding maps with H3 density maps demonstrated that Hrp1 and Med15 are both localized to regions with high H3 density. Analysis of the histone density at selected common Med15 and Hrp1 bound promoters in *Δmed15* did not show any significant changes in H3 density, but instead indicated an increased occupancy by Hrp1, suggesting that Med15 might play a role in removing Hrp1 from specific promoters.

2.2 CONCLUDING REMARKS

Work presented in this thesis has in our opinion contributed to our understanding of the function and organization of the conserved Mediator complex. Our purification strategy allowed us to obtain highly homologous preparations that made possible EM characterization of *S. pombe* Mediator (Elmlund et al, 2006). The isolation of the two various form of yeast Mediator complex that are similar in composition and transcriptional behavior to metazoan Mediators helped to establish the evoluationary conserved structure and function of Mediator complex (Paper I, II). Since the kinase module was not identified as a part of budding yeast Mediator, *S. pombe* Mediator is a good choice to address the architecture of kinase submodule by biochemical means. Analysis of subunit composition of Mediators purified from deletion strains for Cdk8 module components clarified the subunit organization within the kinase module of *S. pombe* Mediator. We demonstrated that core Mediator association with pol II and kinase module are mutually exclusive and that Med13 subunit rather than Cdk8 itself is the key subunit that blocks interaction with pol II (PaperIII).

We have also addressed the possible mechanisms of transition between transcriptionally incompetent L-Mediator and transcriptionally active S-Mediator. We suggest that interaction with non-phosphorylated pol II CTD might contribute in weakening Cdk8 module-S-Mediator contacts and promote kinase module dissociation upon transcription initiation. Other factors (e.g. binding of activators or repressors) might contribute to this transition, perhaps by changing Mediator conformation to alleviate pol II binding or contrary stabilizing the Cdk bound state to promote repression.

While trying to characterize putative Mediator subunit Med15 we were surprised to find it in association with chromatin remodeling protein Hrp1. Perhaps *S. pombe* Mediator association with Hrp1 should not be surprising in the view of emerging

information concerning multiple modes of Mediator action. Our work did not conclusively establish the functional role for Hrp1 in Mediator function, but has opened a door for future studies.

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